Human skin dendritic cells as target for anti-tumor vaccination

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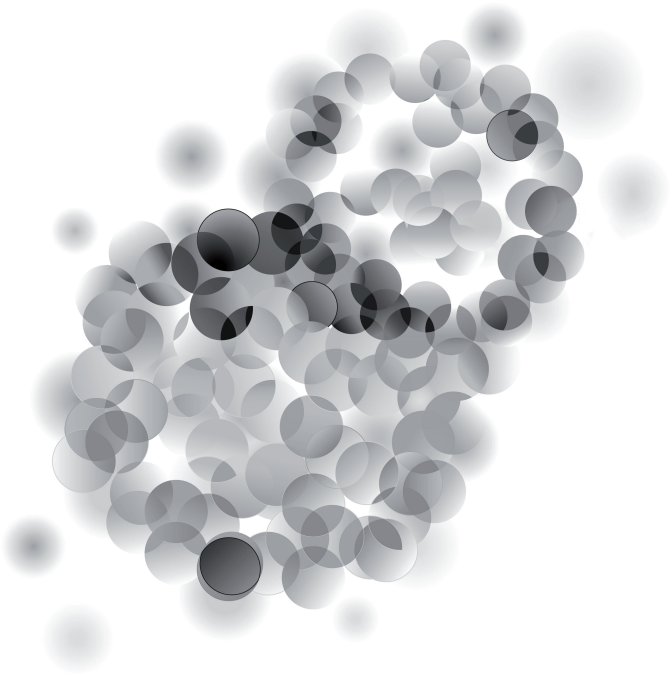

Chapter 4

Langerin-mediated internalization of modified peptides routes antigens to early endosomes and facilitates cross-presentation by human Langerhans Cells

4

Abstract

The potential of the skin immune system to generate immune responses is well established and the skin is actively exploited as vaccination site. Human skin contains several antigen-presenting cell (APC) subsets with specialized functions. Especially the capacity to cross-present exogenous delivered antigens to $CD8^+$ T cells is of interest for the design of effective immunotherapies against viruses or cancer. Here, we show that primary human LCs are able to cross-present synthetic long peptides (SLPs) to $CD8^+$ T cells. In addition, modification of those SLPs using antibodies against the receptor langerin, but not dectin-1, further enhanced the cross-presenting capacity of LCs through routing of internalized antigens to less proteolytic $EEA\text{-}1^+$ early endosomes compared to dectin-1. Coupling of the glycan structures Le^b and Le^y, the natural ligands for langerin, to the SLPs also resulted in langerin-mediated uptake and enhanced cross-presentation. The potency of LCs to enhance $CD8^+$ T cell responses could be further increased through activation of LCs with the TLR3 ligand polyinosinic:polycytidylic acid **(**pI:C). Altogether, the data provide evidence that human LCs are able to cross-present antigens after langerin-mediated internalization. Furthermore, the potential of antigen modification to target LCs specifically provides a rationale to generate effective anti-tumor or anti-viral CTL responses.

Introduction

Antigen presenting cells (APCs) and, in particular, dendritic cells (DCs) induce adaptive immune responses through the presentation of endogenous peptides in the context of MHC class I and exogenous peptides in the context of MHC class II molecules to $CD8^+$ and $CD4^+$ T cells, respectively. In addition, DCs are able of capturing and presenting exogenously derived antigens in MHC class I molecules, a process known as cross-presentation [1]. Antigen cross-presentation plays an important role in the priming of cytotoxic T cells against viruses or tumors, but is also important in maintaining self-tolerance [2]. However, not all DC subsets have identical cross-presentation capacities and some DC subsets appear better equipped for this task. Therefore, careful selection of the DC subset is of utmost importance in the design of anti-tumor or anti-viral DC targeting vaccination strategies.

Langerhans cells (LCs) are a subset of DCs present in mucosal tissues and stratified epithelium, like the epidermis of the skin. Human LCs are characterized by the expression of CD1a, the C-type lectin receptor (CLR) langerin and the presence of Birbeck granules, which are associated with langerin expression [3]. Langerin mediate recognition through the interaction with glycoconjugates such as high-mannose structures, mannan or β-glucans expressed on the surface of pathogens [4]. Langerin mediates ligand internalization for antigen processing and presentation and, therefore, langerin has a potential to be used to specifically deliver antigens conjugated to glycans or α-langerin antibodies to LCs. Although capture of exogenous antigens by human LCs results in the induction of $CD4^+$ T cell responses [5;6] it still remains under debate whether human LCs are able to cross-present exogenous antigens. *In vitro* derived LCs cultured from $CD34^+$ progenitor cells efficiently promoted $CD8^+$ T cell proliferation after internalization of soluble peptides [7]. Also, LCs pulsed with a short EBV peptide or a 39 amino-acid long peptide containing the EBV minimal epitope, were more efficient than pulsed dermal DCs in the cross-presentation of the EBV antigen to memory $CD8^+$ T cells [8]. This enhanced $CD8^+$ T cell activation by LCs was dependent on the interaction between CD70 and CD27 [8], but did not rely on specific, receptor-mediated uptake of antigens. On the other hand, others reported that isolated human LCs were unable to cross-present heat-inactivated measles virus, which was specifically recognized and internalized by langerin [9]. In addition, studies performed in mice also suggest that LCs may have lower cross-presenting capacity [10]. Using a murine model of *Candida albicans* skin infection, the authors showed that LCs were dispensable in the generation of cytotoxic T cells [10]. Instead, $langerin^+$ dermal DCs (dDCs) were required for the generation of antigen specific CTL and Th_1 cells against *C. albicans*. Other studies have recently suggested that cross-presentation is mainly an attribute of the $langerin^+$ dDC subpopulation and not of LCs in murine skin [11;12]. However, the existence of an equivalent of this $langerin^+$ dDC subpopulation in human skin has been questioned[13], although very recently, a $langerin^+$ APC subpopulation has also been detected in the human dermis [14].

Because of their APC-restricted expression pattern and their function as antigen-uptake receptors for processing and presentation, CLRs have often been studied as targeting receptors for vaccination [15;16]. Antigen targeting to DEC-205, DCIR, CLEC9a, dectin-1 and DC-SIGN on DCs resulted in receptor internalization and enhanced antigen-specific $CD4^+$ and $CD8^+$ T cell responses [17-20]. Since CLRs are expressed by specific DC subsets, the choice of a CLR for targeting does not only determine the antigen internalization pathway, but also to which DC subset the antigen is targeted. Various pathways involved in cross-presentation after CLR-mediated antigen internalization of antigens have been proposed. One mechanism involved the translocation of antigen into the cytoplasm for proteosomal degradation, followed by TAP-mediated peptide transport in the endoplasmatic reticulum (ER) and loading onto MHC class I molecules [21;22]. Antigenic peptides can also be generated in the endocytic pathway in a proteosome-independent manner and subsequently bind to recycling MHC class I molecules present within endosomal compartments [23-25]. Recently, it was shown that antigen targeting to specific intracellular compartments, either to early endosomes via CD40 and mannose receptor antibody-conjugates or to late lysosomal compartments via DEC-205, resulted in antigen cross-presentation [26]. Targeting antigens to early endosomes has been shown to result in the most efficient antigen cross-presentation, suggesting that the endocytic compartments to which antigens are delivered determine the efficiency of cross-presentation.

In this study we set out to investigate the role of human LCs in cross-presentation of synthetic long peptides (SLPs) conjugated to antibodies specific for the CLRs langerin and dectin-1, which gave us the opportunity to study the role of each receptor in processing and shuttling of antigens to MHC class I-loading compartments. To determine whether antigen uptake via langerin or dectin-1 by human LCs results in a different intracellular routing and antigen cross-presentation, we analyzed co-localization of both receptors with the early endosomes marker EEA-1 and the lysosomal marker LAMP-1 in pulse-chase experiments. Here, we report that targeting langerin either with antibodies or glycans, but not dectin-1, resulted in enhanced cross presentation. Altogether, these results support the rationale to develop vaccines that specifically target langerin on human LCs to induce anti-tumor $CD8^+$ T cell responses.

Results

LCs are the main langerin$^+$ cells in human skin

Although langerin has been classically reported to be exclusively expressed on epidermal LCs in human skin [27;28], recent publications have challenged this knowledge by reporting the existence of a langerin$^+$ $CD1a^+$ dDC in human dermis, lung, liver and lymphoid tissue [14]. In order to analyze whether langerin expression

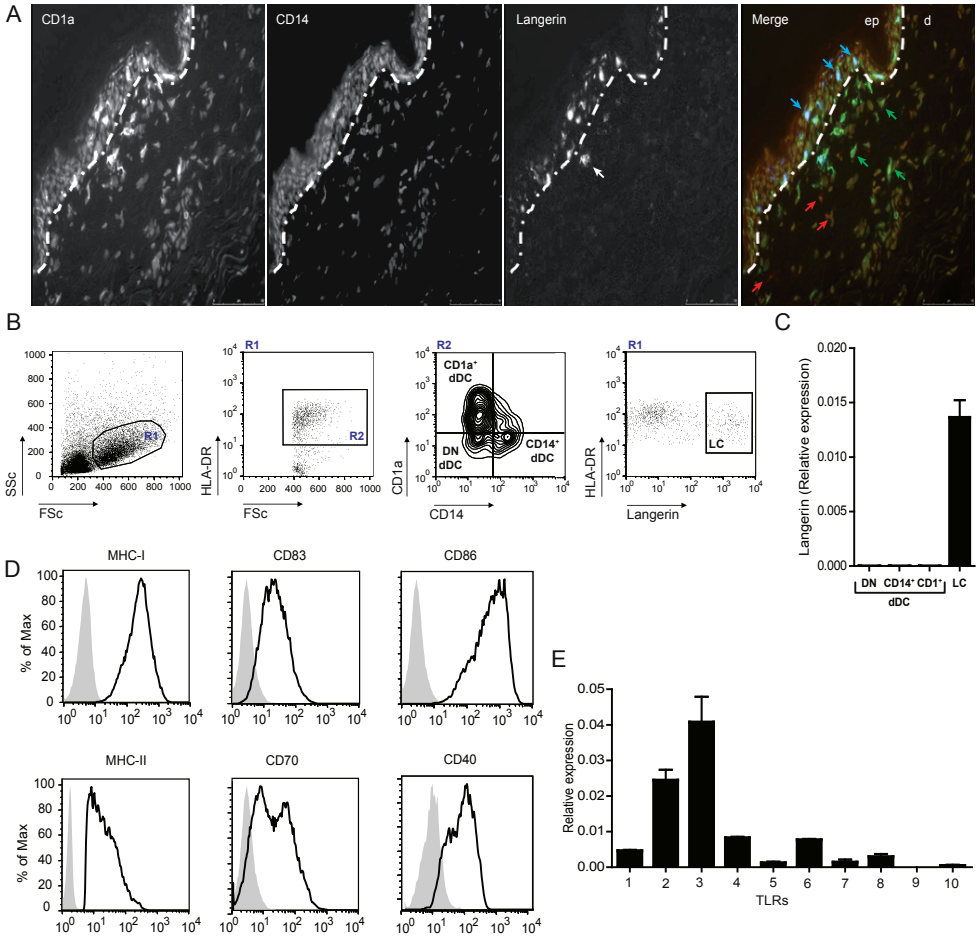

Figure 1. Langerin is exclusively expressed by human LCs. A. Staining of a section of steady-state human skin for langerin (blue), CD1a (green), CD14 (red) and Hoechst (yellow) and analyzed by fluorescence microscopy. B. Gating strategy of FACS-sorted LCs, $CD14^+$, $CD1a^+$ and double negative dermal DCs. C. Langerin mRNA is exclusively expressed in primary, FACS-sorted LCs and not by the other skin DC subsets. N=3, each experiment contains sorted cells of at least 5 skin donors. mRNA values are normalized to GAPDH levels. D. Phenotypical characterization of steady-state LCs. Open histograms: specific antibody; filled histograms: isotype control. Data are representative for three donors. E. Expression profile of the human TLRs on isolated LCs. N=4, each experiment contains sorted cells of at least 5 skin donors. mRNA values are normalized to GAPDH levels.

is restricted to epidermal LCs, human skin sections were stained for CD14, CD1a and langerin. As shown in Figure 1A, the great majority of langerin staining was observed in epidermal LCs, which were also positive for CD1a and only rare $CD1a^+$ $langerin^+$ cells could be observed in the dermis in close proximity with the dermoepidermal junction (Figure 1A). These cells could simply represent dermal DCs that have been

in contact with TGFß leaked from the epidermis, resulting in upregulation of langerin, as demonstrated by Bigley *et al*. [14]. Nevertheless, the frequency of these cells is extremely low and it is questionable whether their presence in the dermis might have any functional relevance. Indeed, most of the dermal CD1a$^+$ DCs observed were devoid of langerin expression (Figure 1). Additionally, we confirmed the data by quantitative RT-PCR analysis for langerin on FACS-sorted HLA-DR$^+$ APCs isolated from the dermis and epidermis (Figure 1B). Figure 1C confirms that langerin is exclusively expressed by LCs and not by the dermal CD1a$^+$ DCs, CD14$^+$ DCs or the HLA-DR$^+$CDa1$^-$CD14$^-$ dermal DC subset, which may be constituted, among others, by macrophages and BDCA3$^+$ skin DCs.

We next investigated the maturational phenotype of the LCs present in human skin. Therefore, LCs were isolated from human skin and analyzed for the expression of

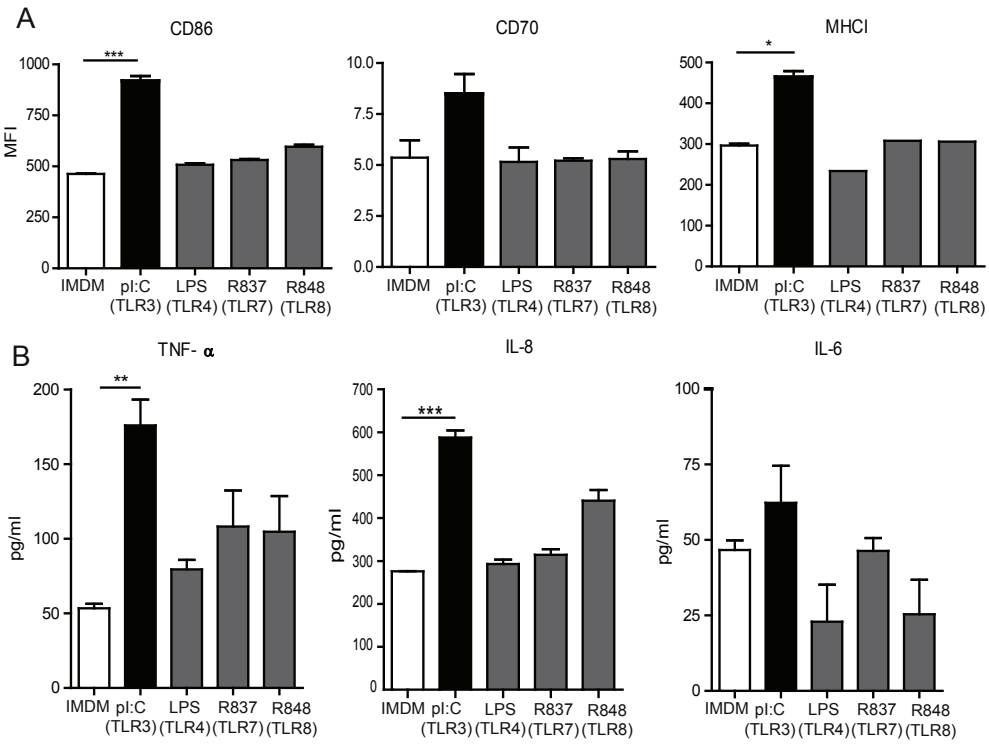

Figure 2. *In vitro* maturation of LCs upon stimulation with the TLR3 ligand pI:C. A. Phenotypical characterization of human LCs after 16 hours of culture in the presence of the indicated TLR ligands. The mean fluorescent intensity is depicted for MHC class I, CD86 and CD70. Data represent average ± SEM of 3 independent skin donors. B. Cytokines produced by human LCs cultured for 16 hours in the presence or absence of indicated TLR ligands. Data represent average ± SEM of 3 independent skin donors.

co-stimulatory and maturation markers. LCs showed expression of co-stimulatory and maturation markers, like CD86, CD83, CD40 and CD70 (Figure 1D). LCs also expressed high levels of MHC class I and II molecules (Figure 1D). In addition, the expression profile of the human Toll-like receptors (TLRs) was analyzed on LCs using quantitative RT-PCR analysis. As shown in Figure 1E, all human TLRs, except TLR9, could be detected in LCs, with TLR3 being the most abundant, as previously described [29;30]. Thus, LCs are the main langerin$^+$ cells in the human skin that express high levels of MHC class I and II molecules and TLR3.

Human LCs mature in vitro upon stimulation with pI:C, but are not affected upon stimulation with other TLR ligands

To test whether the pattern of TLR expression has a functional correlate in terms of responsiveness to specific TLR ligands, we investigated the effects of various TLR-specific compounds on the maturation of LCs and their cytokine responses. As shown in Figure 2A, only the TLR3 ligand pI:C induced an upregulation of the co-stimulatory molecules CD86 and CD70 and MHC class I, while the TLR4 ligand LPS and the TLR7/8 ligands R837 and R848 had no effects (Figure 2A). In addition, only pI:C induced an enhanced production of the pro-inflammatory cytokines TNF-α, IL-6 and IL-8 (Figure 2B). LCs did not secrete the anti-inflammatory cytokine IL-10 either in the presence or absence of pI:C, LPS, R837 or R848 (levels below 10 pg/ml; data not shown). So, human LCs mature upon stimulation with the TLR3 ligand pI:C, as was expected based on the abundant expression of TLR3.

pI:C treatment enhances the cross-presentation capacity of LCs

In order to investigate the capacity of LCs to cross-present SLPs, we pulsed human LCs with a titration of a 16 aa long MART-1 peptide (C-YTTAE*ELAGIGILTV*) containing an HLA-A2-restricted epitope (in italics) recognized by a MART-1 specific CD8$^+$ T cell clone, in the presence of various TLR ligands. This clone responds to specific peptide-MHC-I complexes by the production of IFN-γ. To verify that the 16 aa SLP requires antigen processing before loading on MHC class I molecules, a T2 assay was performed. The data presented in Figure 3A excludes the possibility of direct external loading since the SLP did not stabilize HLA-A2 molecules on the surface of the TAP-deficient T2 cell line (Figure 3A). As a positive control, T2 cells were incubated with the minimal MART-1 epitope that can directly bind and stabilize HLA-A2 on the surface of the T2 cells (Figure 3A). As demonstrated in Figure 3B, human LCs were able to cross-present antigen to the MART-1 specific CD8$^+$ T cell clone as measured by the secretion of IFN-γ. Moreover, simultaneous administration of MART-1 peptide with pI:C resulted in a significant increased activation of the CD8$^+$ T cells, whereas addition of LPS, R837 or R848 did not enhance cross-presentation (Figure 3B). Thus, human LCs are able to cross present soluble SLPs, which could be further enhanced in the presence of pI:C.

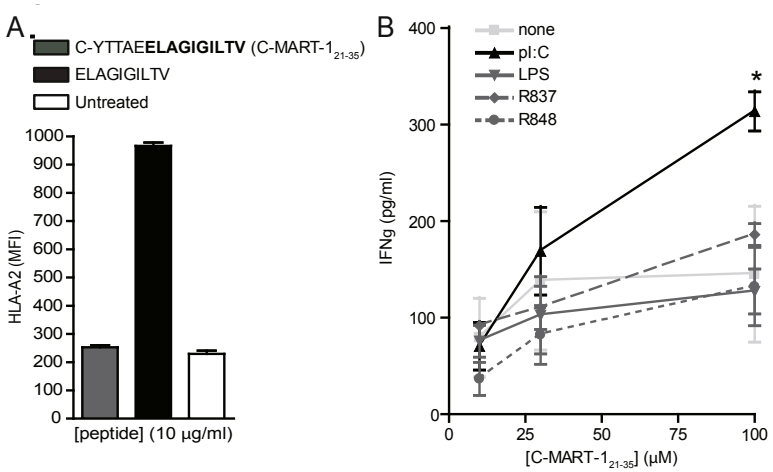

Figure 3. pI:C stimulation of LCs results in superior antigen cross-presentation. A. 1x10^5 T2 cells were incubated with a 16 aa long MART-1 peptide, the minimal CD8 epitope or no peptide, where after the cells were analyzed for the expression of surface HLA-A2 by flow cytometry. Data represent average of three ± SD. B. Human LCs were incubated with the synthetic long MART-1 peptide for 3 hours and indicated TLR ligands, washed and co-cultured with a MART-1 specific CD8$^+$ T cell clone. After 24 hours of co-culture, T cell activation was measured by IFN-γ ELISA in the supernatants. Data of one representative experiment measured in triplicate is shown, n=3.

Targeting langerin, but not dectin-1, using SLP-antibody conjugates enhanced antigen cross-presentation in LCs

Since human LCs have the capacity to cross-present MART-1 SLPs, which were presumably internalized by pinocytosis, we next investigated whether receptor-mediated internalization would enhance cross-presentation by altering the endocytic routing of the antigens. To investigate this, anti-langerin or anti-dectin-1 mouse monoclonal antibodies were conjugated to the MART-1 SLP via maleimide-thiol coupling through the N-terminal Cys on the SLP. Both langerin and dectin-1 are expressed on human LCs (Figure 4A) and, as shown in Figure 1, langerin is a specific marker of human LCs, making this receptor a suitable target for LC-targeting immunotherapy. Conjugation of the SLPs to anti-langerin antibodies resulted in significantly higher activation of the CD8$^+$ T cell clone (Figure 4B) as compared to the SLP alone or conjugated to the anti-dectin-1 antibody or an isotype control, indicating that langerin-targeting enhances cross-presentation by human LCs. Furthermore, langerin-specific responses were further increased when LCs were simultaneously exposed to pI:C (Figure 4C). Interestingly, although both dectin-1 and langerin were expressed on LCs (Figure 4A), we could not detect any dectin-1-specific enhancement on cross-presentation as compared to the isotype control (Figure 4b and C), suggesting that antigens endocytosed via dectin-1 follow an intracellular routing that did not result in proper processing and/or loading to MHC class I. Together, these data show that only langerin targeting of MART-1 SLPs

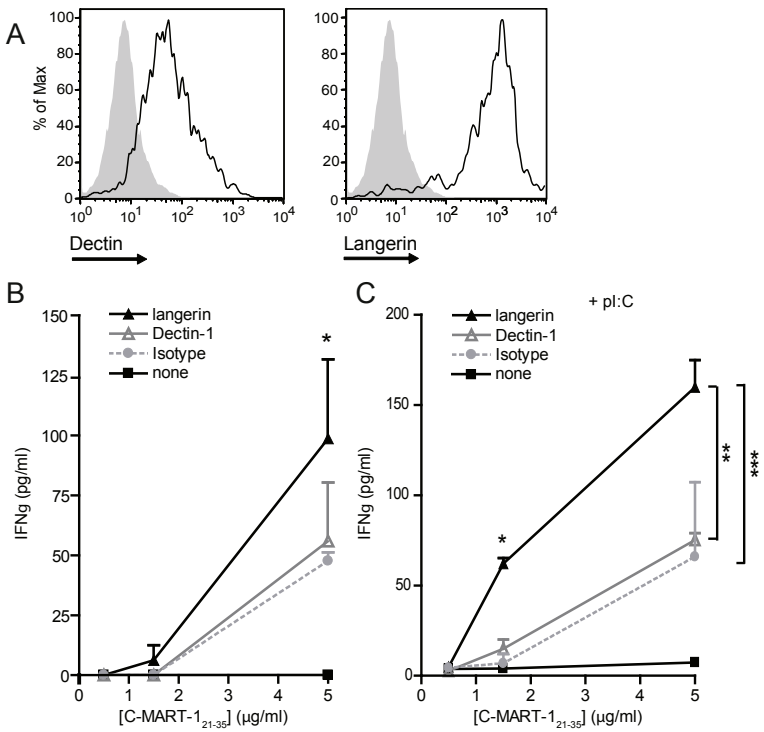

Figure 4. Uptake of SLPs conjugated to langerin route to early endosomes and results in enhanced cross-presentation to antigen specific CD8⁺ T cells. A. Dectin-1 and langerin are highly expressed by human LCs as measured by flow cytometry. Open histograms: specific antibody; filled histograms: isotype control. Data are representative for 3 donors. B. MART-1 (CYTTAEELAGIGILTV) peptides were conjugated to anti-langerin and anti-dectin-1 or mIgG1 isotype control mAbs and incubated with human LCs for 3 hours in the presence or absence of 20 µg/ml pI:C, and co-cultured with a MART-1 specific CD8⁺ T cell clone. Activation of the T cells was measured by IFN-γ ELISA on the supernatants taken after 24 hours of co-culture. Data are representative of two independent experiments and depict average ± SEM of triplicates.

allowed antigen routing to a cross-presentation compartment, which could be enhanced by simultaneous triggering of TLR3.

Langerin routes antigens to specific early endosomes

To investigate the differences in antigen cross-presentation after langerin and dectin-1 targeting, we pulsed LCs using fluorescently-labeled antibodies and followed their intracellular localization using the early endosomal marker EEA-1 and the lysosomal marker LAMP-1 by imaging flow cytometry. The fluorescence associated to the anti-langerin antibody hardly decreased over time (Figure 5A), while that of the anti-dectin-1 antibody rapidly decreased (Figure 5B), suggesting that langerin routes antigens to a less degradative compartment as compared to dectin-1. We then investigated the co-localization of each of the CLR-specific antibodies with EEA-1 $^{+}$ compartments and observed that dectin-1 had a longer and

stronger association with EEA$^+$ compartments compared to langerin based on the co-localization score being close to 1 (Figure 5C and D). The co-localization between langerin and LAMP-1$^+$ compartments or dectin-1 and LAMP-1$^+$ compartments was poor and did not differ in time and intensity (Figure 5C and D).

Since there was a divergence in the degradation of the fluorescence signal and the localization to early endosomes, we speculated that dectin-1 and langerin routed to different types of early endosomes with differing degradative capacities. To investigate this, we used a 3-color co-localization feature and addressed the co-localization of either EEA1 or LAMP1 with dectin-1 and langerin. In resting LCs, both dectin-1 and langerin are expressed on the membrane of LCs, as shown in Figure 4A. However, there was a significant proportion of both receptors localized in an intracellular compartment, which appeared to be EEA1$^+$, but not LAMP1$^+$ (Figure 5E). Presumably, this compartment serves as an intracellular depot for quick up regulation of both receptors on the membrane of LCs. Interestingly, when membrane-located dectin-1 and langerin are triggered, they follow different endocytic pathways, as can be inferred from the low 3-color co-localization scores observed (Figure 5E). These data indicate that langerin routes to specific EEA-1$^+$

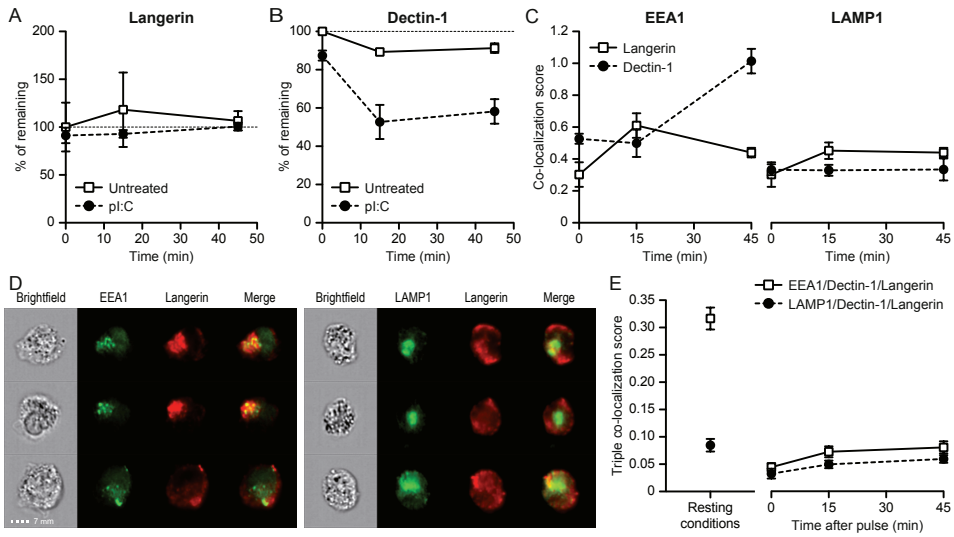

Figure 5. Langerin routes to less degradative endosomal compartments in LCs after antigenic pulse compared to dectin-1. A+B. Remaining fluorescence of anti-langerin (A) and anti-dectin-1 antibodies (B) in a pulse-chase in the presence or absence of pI:C. C. Co-localization of langerin and dectin-1 with the intracellular compartments EEA-1 and LAMP-1 after receptor binding and internalization at the cell surface. D. Representative examples of cells showing moderate co-localization of langerin with EEA-1 and poor co-localization of langerin with LAMP1. E. Three color co-localization score of langerin, dectin-1 and EEA-1 or LAMP-1 in resting conditions (left panel) or after receptor triggering at the cell surface (right panel) measured by imaging flow cytometry.

early endosomes that, presumably, are better equipped to facilitate cross-presentation than the early endosomes where dectin-1 routes to.

Conjugation of SLPs with langerin-specific glycans enhanced uptake and cross-presentation

CLRs recognize specific glycan motifs on pathogens or glycoproteins, allowing the internalization of the bound pathogen or antigen. Glycans offer several advantages over antibodies in DC/LC targeting strategies for immunotherapy, mainly related to their small size, synthetic possibilities and poor immunogenicity. Langerin has been described to have specificity for Lewis-type glycans and several types of high-mannose glycans[31]. Indeed, as can be observed in Figure 6A, a soluble langerin-Fc chimeric construct efficiently recognized Le^Y epitopes on a polyacrylamide scaffold. To a lesser extent, Le^b glycans were also recognized, but no binding could be detected to Le^X, as recently reported [32]. No differences in coating efficiency between the glycans were observed, as measured by an streptavidin ELISA (data not shown).

Conjugation of the MART-1 SLP using Le^b or Le^Y resulted in enhanced cross presentation by the LCs to the $CD8^+$ T cell clone as measured by IFN-γ production, compared to unmodified or Le^X-coupled MART-1 SLP (Figure 6B). As expected, the simultaneous stimulation of LCs with pI:C further increased the production of IFN-γ by the $CD8^+$ T cells, suggesting that cross-presentation is enhanced through TLR3 signaling (Figure 6C). Furthermore, glycosylated SLPs exerted their action in a langerin-specific manner, as the effects could be inhibited using a blocking antibody against langerin. Since glycans are chemically small structures, the residual stimulation of the $CD8^+$ T cell clone might be explained by non-specific uptake of the glycopeptides, possibly via pinocytosis. In summary, conjugating Le^Y glycans to SLPs facilitates targeting to langerin and increases cross-presentation by human LCs.

Discussion

Within the human skin, at least four main and distinct populations of DCs can be identified, namely LCs and $CD14^+$, $CD1a^+$ and $BDCA3^+$ dermal DCs. After activation, it has been shown that the $langerin^+$ LCs and dDC subsets are able to migrate to the skin-draining lymph nodes, where they activate $CD4^+$ and $CD8^+$ T cell responses [33;34]. The precise function of each subset is still under debate, especially in humans. In particular, the capacity of each subset to cross-present antigens is not well defined, which is of great importance for the development of effective therapeutic (anti-cancer) vaccines. In this study, we have investigated the cross-presentation capacity of human LCs when SLPs were targeted to distinct CLRs. Specific targeting of SLPs to the LC-specific receptor langerin, using either antibody- or glycan-modification, resulted in enhanced activation of effector $CD8^+$ T cells.

These results imply that human LCs are a suitable candidate for *in vivo* targeting of vaccines. Although LCs are located in the epidermis of the human skin, administration of a LC-targeting vaccine can also be applied intradermally, since intradermally deposited DEC-205 or langerin antibodies have been shown to be rapidly captured by LCs [35].

In our study, targeting peptides to langerin using monoclonal antibodies resulted in efficient cross-presentation. In contrast, antigens internalized via the CLR dectin-1 did not allow enhanced cross-presentation, showing that the intracellular routing of antigens internalized via dectin-1 is different from that of langerin in LCs. This is

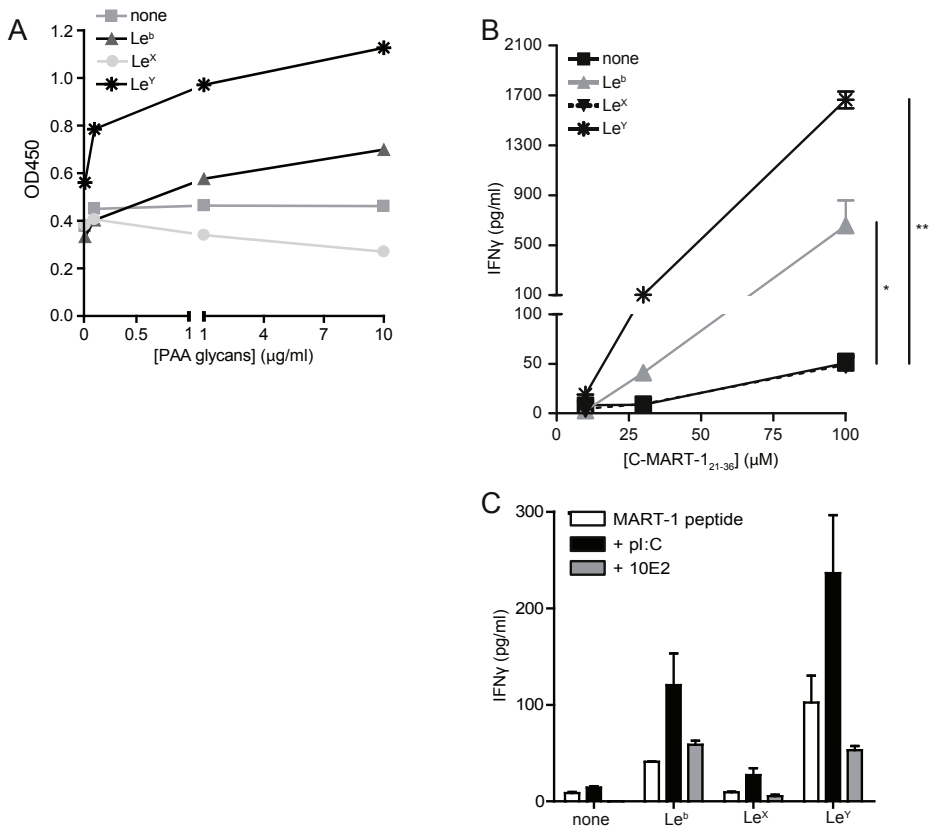

Figure 6. Le^b and Le^y glycan conjugation to MART-1 SLPs results in langerin-mediated uptake and enhanced cross-presentation by LCs. A. Binding of langerin-Fc constructs to PAA-coupled glycans was measured by ELISA. Data are representative of three independent experiments. B+C. Human primary LCs were incubated with the depicted glycan- conjugated peptides at different concentrations for 3 hours, followed by the addition of MART-1 specific CD8$^+$ T cells. After 24 hours of co-culture, IFN-γ secretion was measured in the supernatants using ELISA. Data are representative for three independent experiments and depict average ± SD of triplicates. C. Simultaneously with the addition of peptides, LCs were incubated with 20 µg/ml pI:C with or without a blocking antibody to langerin (20 µg/ml) to specify the langerin-mediated uptake of the glycan-modified SLPs.

supported by our imaging flow cytometry data, which demonstrates that dectin-1 routes to more degradative intracellular compartments which also did not co-localize with langerin^{+}EEA-1^{+} compartments. However, it has been shown for *in vitro* generated moDCs that dectin-1 allowed binding and internalization of CMV pp65 expressing apoptotic cells and this interaction resulted in cross-presentation to CMV pp65 specific CD8^{+} T cells [19], showing the potential of dectin-1, when expressed on DC, to facilitate routing of antigen to MHC class I loading compartments. The discrepancy between these results and the findings presented in this paper concerning cross-presentation after dectin-1 targeting, might be explained by the difference in cell types, primary LCs versus *in vitro* generated DCs. Alternatively, it may also be attributed to the mode of antigen delivery. In the experiments described here, dectin-1 was targeted using monoclonal antibodies conjugated to soluble MART-1 peptides, whereas in the study of Weck *et al.* dectin-1 facilitated the uptake of total apoptotic cells [19].

Dectin-1 is not the only CLR in which the capacity to cross-present is in part determined by the mode of antigen delivery. Van der Vlist *et al.* reported that human LCs were not able to cross-present antigens derived from whole measles virus (MV) or MV-infected apoptotic cells taken up via langerin [9]. A reasonable explanation for these opposite findings could be the difference in size between the antigen formulation used in their study (whole MV and MV-infected cells) compared to SLPs used in our studies. Further experiments should be performed to clarify whether the size of antigens taken up via langerin influences the internalization, routing and cross-presentation capacity of LCs.

This study also showed that cross-presentation by human LCs was enhanced in the presence of the TLR3 agonist pI:C. The combined administration of antigens and TLR agonists is reported to be necessary to prevent the induction of T cell tolerance. Recently the requirement of a potent activator to overcome the tolerogenic state of LCs to selectively and specifically induced the activation and proliferation of skin resident regulatory T (Treg) cells has been described [36]. Similar as DCs, in the presence of danger signal derived from pathogens, LCs become activated and induced the proliferation of effector memory T cells present in the skin and reduced the activity of Tregs [36]. It seems likely that the precise function of LCs *in vivo* might be determined by danger stimuli derived from the microenvironment [37].

Altogether, we have shown the capacity of human primary LCs to cross-present soluble synthetic long MART-1 peptides. Additionally, cross-presentation by LCs was enhanced when cells were concomitantly matured using the TLR3 agonist pI:C. Targeting of MART-1 to langerin using antibodies or glycans resulted in further enhancement of the activation of MART-1 specific CD8^{+} T cells through langerin-mediated routing of antigens to less proteolytic early endosomes compared to dectin-1-mediated antigen internalization. These results provide a rationale for the development of new *in-vivo* targeting vaccines that target human LCs via langerin for the induction of effective anti-tumor or anti-viral CTL responses.

Materials and methods

Cells

Primary, human LCs were isolated from abdominal resections from healthy donors undergoing cosmetic surgery (Bergman Clinics, Bilthoven, The Netherlands) and were obtained with informed consent within 24 hours after surgery as previously described [38]. Shortly, 5 mm thick slices of skin, containing the epidermis and dermis, were cut using a dermatome. The slices were incubated in dispase II (1 mg/ml, Roche Diagnostics) in IMDM (Invitrogen) supplemented with 10% FCS (BioWhittaker), 50 U/ml penicillin (Lonza), 50 µg/ml streptomycin (Lonza) and 10 µg/ml gentamycin (Lonza) overnight at 4°C followed by mechanical separation of dermis and epidermis using tweezers. The epidermis was washed in PBS, cut into small pieces and incubated in PBS containing DNase I (200 U/ml, Roche Diagnostics) and trypsin (0.05%, Invitrogen) for 30 minutes at 37°C. After incubation, a single cell suspension was generated using 100 µm nylon cell strainers (BD Falcon) and cells were layered on a Ficoll gradient. An average of $1x10^4$ LCs per cm^2 of tissue with a purity higher than 90% were obtained and characterized as $CD1a^+$ $langerin^+$ cells by flow cytometry as described below. LCs were cultured in IMDM supplemented with 10% FCS, 50 U/ml penicillin, 50 µg/ml streptomycin and 10 µg/ml gentamycin. When indicated, LCs were matured in the presence of 20 µg/ml pI:C (Invivogen), 20 ng/ml LPS (derived from *E. coli*, Sigma), 5 µg/ml R837 (Invivogen) or 5 µg/ml R848 (Invivogen). When indicated, LCs were obtained by spontaneous migration. Shortly, epidermis and dermis were separated as described above. The epidermis was washed in PBS and cultured for 2 days in a 25 cm^2 culture dish (Greiner) containing 40 ml of IMDM supplemented with 10% FCS, 50 U/ml penicillin, 50 µg/ml streptomycin and 10 µg/ml gentamycin to allow spontaneous migration of LCs. After 2 days, cells present in the supernatant were harvested and layered on a Ficoll gradient and further cultured as described above.

Tap-deficient T2 cell line

The TAP-negative BxT hybrid cell line 1.74xCEM (referred to as T2) was used for peptide stabilization assays [39]. T2 cells ($1x10^5$) were incubated overnight with peptides in RPMI at 37°C, washed twice in PBS and surface MHC class I expression assayed by flow cytometry (FACSCalibur, Becton Dickinson) using an HLA-A2 specific monoclonal antibody (Becton Dickinson).

Flow cytometry

Phenotypical analysis of isolated LCs was performed by flow cytometry. Cells were washed in PBS supplemented with 1% bovine serum albumin (BSA) and 0.02% NaN_3 and incubated for 30 min at 4°C in the presence of appropriate dilutions of fluorescent-conjugated mAbs to CD1a (APC, clone HI149, Becton Dickinson), CD14 (FITC, clone MoP9, Becton Dickinson), CD70 (PE, clone Ki-24, Becton Dickinson),

CD86 (PE, clone 2331, Becton Dickinson), HLA-DR (PerCP, clone L203, Becton Dickinson), HLA-ABC (FITC, clone W6/32, ImmunoTools) or CD83 (PE, clone HB15e, Beckman Coulter Immunotech), or corresponding isotype-matched control mAbs (Becton Dickinson). HLA-A2 status of the cells was determined using a specific mAb (Becton Dickinson). The cells were subsequently analyzed using the FACSCalibur and FlowJo software (Tree Star).

Imaging flow cytometry

Approximately $0.1x10^6$ primary human LCs, which were migrated spontaneously from human skin, were incubated for 3 hours at 37°C in culture medium with or without 20 µg/ml pI:C to induce maturation. Cells were then washed twice and incubated in ice-cold culture medium. As indicated, AF647-labeled dectin-1 (AbD Serotec) and PE-labeled langerin (R&D Systems) were added and incubated for 15 min at 37°C in order to allow binding to cell surface expressed langerin and dectin-1. Cells were then washed in ice-cold medium, transferred to a 37°C incubator and samples were obtained at the indicated time points. Cells were then washed in ice-cold PBS and fixated in ice-cold 4% PFA in PBS for 20 minutes. To prevent cell loss during the staining procedure, LCs were mixed with monocyte-derived dendritic cells. Cells were then permeabilized in 0.1% saponin (Sigma) in PBS for 30 minutes at room temperature and subsequently blocked using PBS containing 0.1% saponin and 2% BSA for 30 minutes at room temperature. Stainings were performed at room temperature in PBS supplemented with 0.1% saponin and 2% BSA. After staining, cells were washed twice in PBS, resuspended in PBS containing 1% BSA and 0.02% NaN_3 and kept at 4°C until analysis. Cells were acquired on the ImageStream X (Amnis) imaging flow cytometer. A minimum of 15000 cells was acquired per sample at a flow rate ranging between 50 and 100 cells/second at 60x magnification. At least 2000 cells were acquired from single stained samples to allow for compensation (Supporting Figure 1A and 1B). Analysis was performed using the IDEAS v6.1 software (Amnis). Cells were gated based on the Gradient RMS (brightfield) feature, which was used to select for cells in focus (Supporting Figure 1C and 1D) and langerin expression (Supporting Figure 1E and 1F). Co-localization was calculated using the features bright detail similarity R3 (for 2-color co-localization) or bright detail co-localization 3 (for 3-color co-localization).

Immunofluorescence microscopy

Human tissue sections (7 µm) were fixed in acetone and blocked with goat serum prior to staining. Antibodies directed against CD14, CD1a or langerin were added at 10 µg/ml in PBS containing 1% BSA for 60 min. at 37°C followed by secondary Alexa 488-conjugated rabbit anti-mouse IgG2A, Alexa 546-conjugated rabbit anti-mouse IgG2B or Alexa 647-conjugated rabbit anti-mouse IgG1 specific antibodies (Molecular Probes) for 30 min. at room temperature. Sections were counterstained using hoechst and analyzed by fluorescence microscopy (Leica microsystems).

Modification of MART-1 and Gp100 peptides with glycans and antibodies

The synthetic long peptides MART-1 (CYTTAEELAGIGILTV) and Gp100 (YLEPGPVTANRQLYPEWTEAQRLDC) were produced by solid phase peptide synthesis using Fmoc-chemistry with a Symphony peptide synthesizer (Protein Tecnologies Inc., USA). Peptides were coniugated to glycans and antibodies on the N-terminal cysteine through a tiol-maleimide reaction. To this end, glycans were activated with the use of the bifunctional crosslinker MPBH (4-N-Maleimidophenyl butyric acid hydrazide, Thermo Scientific) and antibodies through reaction with SMCC (Succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate, Thermo Scientific).

First, the hydrazide moiety of MPBH was covalently linked to the reducing end of the glycan via reductive amination. Shortly, a mixture of MPBH (3 eq.), glycan (1 eq.) and picoline-borane (10 eq.) dissolved in Dimethyl-sulfoxide/Acetic acid (7:3, anhydrous and glacial respectively, Sigma) reacted for 2h at 65°C. After cooling down to room temperature, 4 volumes of dichloromethane (Biosolve) were added and the mixture was vortexed thoroughly. Subsequently, 4 volumes of diethyl ether (Biosolve) were added and incubated until glycan-MPBH had completely precipitated. MPBH-glycans were pelleted by centrifugation (2 min at 14000g), then the supernatant was discarded and the pelleted carbohydrate-MPBH was washed with cold diethyl ether 3 times. The obtained glycan-MPBH pellet was resuspended in aqueous 0.1% TFA (trifluoroacetic acid, Sigma) and lyophilized, followed by purification over a 22 x 250 mm Vydac MS214 prep C18 column (Grace Alltech, elution water/acetonitrile, gradient 3% to 50% of acetonitrile in 40 min) on a Dionex prep 3000 HPLC system. The fractions containing the glycan-MPBH were pooled and lyophilized.

Peptides were glycosylated on their terminal cysteines with the activated glycan through a thiol-ene reaction. Briefly, the peptides (3 eq.) were dissolved in 0,05M phosphate buffer (pH 6.5) and added to the carbohydrate-MPBH (1 eq.). After 2 h of reaction at room temperature, the glycosylated peptides were purified using and Vydac MS214 prep C18 columns 10 x 250 mm (Grace Alltech, elution water/acetonitrile, gradient 10% to 50% of acetonitrile in 40 min). The fractions containing the glycopeptide were pooled and lyophilized. The derivatization and purity of the glycosylated peptides was confirmed by HPLC (Vydac 218MS C18 5um 4.6 x 250 mm, Grace Alltech) and MS spectrometry (LCQ-Deca XP Iontrap Thermo Finnigan mass spectrometer in positive mode using nanospray capillary needle). Conjugation of the glycans to the glycopeptides was also confirmed by ELISA using antibodies specific for Leb, LeX and LeY, as previously described[18].

Peptides were conjugated to antibodies using the bifunctional crosslinker Succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC, Thermo Scientific). Briefly, antibodies were activated with SMCC (8 eq.) in phosphate buffer pH 8.2 for 30 min at room temperature. After desalting over a G-25 10X100 mm single use desalting gel filtration column (Amersham), peptides were dissolved in

DMSO and added to the vial containing the antibody. After performing the coupling reaction at room temperature for 2h, the un-conjugated peptide was removed through size-exclusion chromatography using a superdex 75 column (30 x 100 mm, Amersham Biotech) eluting with 50 mM ammonium formate buffer, pH 6.8. The fractions containing the antibody-peptide constructs were pooled and lyophilized.

Generation of langerin-Fc constructs
The binding capacity of Langerin to various Lewis-type glycans was determined using langerin-Fc molecules. Langerin-Fc was generated by amplifying the extracellular domains of langerin (aa 63–328) on RNA of LCs by PCR. The product was confirmed by sequence analysis and fused at the C-terminus to human IgG1-Fc in the Sig-pIgG1-Fc vector. Langerin-Fc was produced by stable transfection of CHO cells and Langerin-Fc concentrations were determined by ELISA.

ELISA-based langerin binding assay
The conjugation of the Le^b, Le^X and Le^Y glycans to the MART-1 peptides was confirmed by ELISA using anti-Le^b, -Le^X and -Le^Y antibodies (Calbiochem)[18]. Briefly, glycopeptides were dissolved in PBS containing 0.05% BSA and coated onto NUNC maxisorb plates (Roskilde) and incubated overnight at 4°C. Plates were blocked with 1% BSA in PBS to avoid non-specific binding. After extensive washing, the glycopeptides were incubated with langerin-Fc for 90 min at RT. Binding was detected using a peroxidase-labeled F(ab')2 goat anti-human IgG/Fcγ specific antibody. Signal detection was achieved by incubation with 1.3 mM H_2O_2 in the presence of TMB (3,3',5,5'-tetramethylbenzidine) in 0.1M sodium acetate-citrate buffer until the development of the reaction. The reaction is then stopped using 1 M H_2SO_4 and absorbance is measured at 450 nm using a colorimeter (BioRad). As a positive control, biotin-labeled Le^b, Le^X and Le^Y conjugated to polyacrylamide (PAA; Lectinity) were used.

Quantitative real-time RT-PCR
Cells were lysed and mRNA was isolated using an mRNA Capture kit (Roche). cDNA was synthesized using the Reverse Transcription System kit (Promega) following manufacturer's guidelines. Each experiment contained cells isolated from at least 5 skin donors to obtain sufficient cells numbers for analysis. Oligonucleotides were designed using the Primer Express 2.0 software (Applied Biosystems) and synthesized by Invitrogen Life Technologies. Real-Time PCR analysis was performed as previously described using the SYBR Green method in an ABI 7900HT sequence detection system (Applied Biosystems)[40]. GAPDH was used as an endogenous reference gene.

Antigen presentation to human $CD8^+$ T cell clones specific for MART-1
A $CD8^+$ T cell clone specific for MART-1_{26-35} was generated and cultured as described

previously [41]. The modified 16 aa long MART-1_{21-35} peptides (C-YTTAEELAGIGILTV) were added to 20.000 HLA-$A2^+$ LCs, obtained after migration from the skin, per well at indicated concentrations together with poly I:C (20µg/ml) for 3 hours at 37°C. After extensive washing, HLA-$A2^+$ MART-1 specific $CD8^+$ T cells (100.000/well) were added to the wells. After 24 hrs, supernatants were taken and IFN-γ levels were measured by sandwich ELISA using specific antibody pairs from Biosource and according to the manufacturer's guidelines.

Statistical analysis

Results were analyzed using either a one-way ANOVA followed by Bonferrroni Multiple Comparison test or a two-way ANOVA followed by Bonferroni Multiple Comparison test using GraphPad Prism software (GraphPad Software, San Diego, CA). Results were considered to be statistically significant when $p<0.05$.

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Conflict of interest

The authors declare no financial or commercial conflict of interest.

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S1

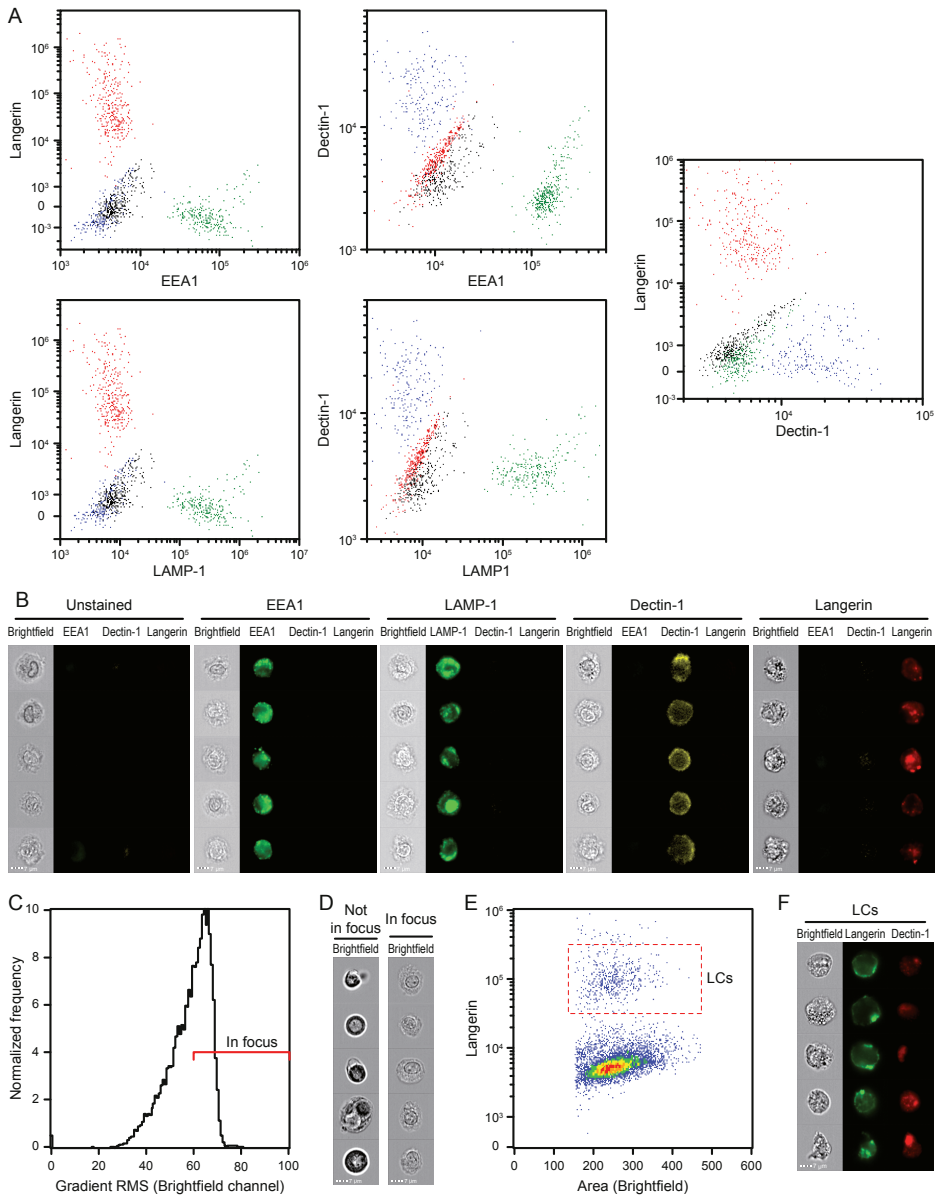

Figure S1. Experimental details of the imaging flow cytometry. A, B. Once the compensation tables were derived, single staining controls were used to verify that compensation was correct by using classical bivariate plots (A) and examining the cell imagery (B). C. Only cells that were properly focused were selected for further analysis based on the feature Gradient RMS applied to the brightfield channel. D. The gate for properly focused cells was verified by examining the cell imagery. E. Non-LCs were gated out by selecting the cells that expressed langerin. F. LCs expressed both langerin and dectin-1.